

REMARKS

This Amendment is supplemental to the Amendment and Reply filed June 3, 2002, in response to the Office Action dated December 2, 2001. Entry of the foregoing and reconsideration on the merits pursuant to 37 CFR 1.112 is respectfully requested.

The application has been amended as set forth above. The attached appendix provides marked-up versions of the amended paragraphs and claims showing the changes that are made.

Amendment of the Text of the Specification:

Line 5 of page 20 is amended to state that "...a significant application of the subject PGCs, which can be maintained in culture for long periods, is for the production of transgenic chimeric avians." The word "transgenic" is added in order to describe the claimed invention more clearly, in accord with the sentence that follows the amended line on page 20: "This will be accomplished by introducing a desired DNA sequence into the cultured PGCs."

Lines 17 and 18 of page 20 are amended to state that " ... a DNA will be introduced that comprises [encodes] a desired gene, e.g., a gene that encodes therapeutic polypeptide, growth factor, enzyme, etc., under the regulatory control of sequences operable in avians. This revision brings the language used to describe the invention into accord with the dogma that a gene encodes a polypeptide, and is submitted in order to describe the claimed invention more clearly. Support for the introduction of a DNA that comprises a desired gene that encodes a polypeptide is found in the specification, for example, in the paragraphs beginning at lines 17 and 25 of page 20, which describes introducing a DNA with a desired gene for a polypeptide such as a therapeutic polypeptide, growth factor, or enzyme, the coding sequences of which are functionally linked to gene expression regulatory sequences that are operable in avians, plus easily detected marker DNA (Page 21, lines 1-4).

The foregoing amendments to the specification do not add new matter.

Amendment of the Claims:

Claims 13 and 18 are further amended to recite that the nucleic acid comprises a polypeptide-encoding sequence that is functionally linked to gene expression regulatory sequences that are operable in an avian cell, support for which is found at page 20, lines 16-24 ("DNA... that encodes a ...polypeptide ... under regulatory control of sequences operable in avians"). The claims are also amended to remove the limitation of expression in an egg, so that the claims encompass transfection with a gene expression construct that may be expressed in tissues of either an embryonic or a hatched transgenic avian, as described, for example, at page 11, lines 16-24 of the specification.

Claims 23 and 26 are further amended, responsive to the Office Action mailed December 2, 2001, to correct typing errors.

Claim 23, along with claims 13 and 22, is intended to depend on claim 12, which recites a method comprising transfecting or transforming the EG cells obtained by the method of claim 1 with a nucleic acid sequence. In their dependence on claim 12, amended claims 13, 22, and 23 are intended to be analogous to amended claims 18, 19, and 21, which depend in like fashion on amended claim 17.

Claim 26 is amended to make it clear that the claim is directed to a method of producing chimeric avians that are germline and somatic cell chimeric avians, support for which is found at page 22, lines 20-21 of the specification..

The above-described amendments of the claims do not add new matter.

Regarding Rejection of the Claims Under 35 U.S.C. §112, First Paragraph:

The amended claims are in compliance with 35 U.S.C. §112, first paragraph.

Claims 1-23 and 25-30 were rejected under 35 U.S.C. §112, first paragraph, on the grounds that the specification does not enable identifying avian EG cells in a mixed cell

population, using chimeric avians that are not germline chimeras, stably transfecting avian cells, or making chimeric avians that express exogenous proteins or have an altered, non-wild phenotype.

Applicants submit that the amended claims do not recite identifying avian EG cells in an avian cell population, so that this ground of rejection is moot.

The Applicants respectfully traverse the ground of rejection that the claims are not enabled because the specification does not disclose uses for somatic chimeric avians that are not germline chimeras. The specification does not have to disclose, and preferably omits, that which is well known by those in the art. In re Buchner, 929 F.2d 660, 661; 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); Hybritech, Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1384; 231 USPQ 81, 94 (Fed. Cir. 1986); see also M.P.E.P. § 2164.01. Those skilled in the art recognize that each chimeric avian produced by injection of pluripotent EG cells into a recipient embryo is unique. The EG cells may give rise to both somatic and germ cells, so that the chimeric avian is a germline chimera, or they may give rise to one or more somatic tissues, but fail to provide germ cells, so that the chimeric avian is a somatic, non-germline avian. The claimed method inherently produces both type of chimeric avians, and the claims properly encompass a method that results in both type of chimeras. The specification provides detailed description of making and using germline chimeric avians because there is great commercial interest in developing methods for producing these. While production of somatic chimeric avians that are not germline chimeras does not have the same commercial importance, one skilled in the art nonetheless would know that methods for making somatic chimeric avians are useful, for example, for studying avian development and the interactions of genetically different cell types within an individual avian. The interest of those in the art in such studies is shown by Watanabe et al. (Development, 1992, 114(2):3331-338, abstract attached), who described making somatic chimeric avians by inserting blastoderm cells into

recipient avian embryos, and studying the chimeric avians to determine the relationship between the type of tissue formed by the injected cells, and the stage of the recipient embryo at the stage of injection. In addition to using somatic chimeras to study embryonic development and cell-cell interactions, those skilled in the art would know that somatic chimeric avians may be used for the same purposes as non-chimeric birds; e.g., as food. Accordingly, the Applicants respectfully request reconsideration and withdrawal of the rejection under 35 U.S.C. §112, first paragraph, on the ground that the specification does not teach uses for non-germline chimeric avians produced according to the claimed invention.

The Applicants respectfully traverse the assertion in the Office Action that the specification does not enable the claimed methods comprising stably transfecting avian cells and making chimeric avians that express exogenous proteins or have an altered phenotype. These grounds of rejection are similar to the grounds for rejection of claims 12-13, 17-19, and 23 under 35 U.S.C. §112, first paragraph, stated on pages 5-6: (a) that the specification does not enable the use of transiently transfected avian EG cells, and (b) that neither the specification nor the art available at the time of filing teach how to use the transfected PGCs to produce transgenic avians that have an altered phenotype or that make exogenous proteins that can be isolated, which the Office Action asserts are the only uses for transfected PGCs disclosed in the specification.

In support of the rejection of the claims under 35 U.S.C. §112, first paragraph, as being non-enabled, the Office Action refers to the Applicants' disclosure in the specification that stably transfected PGCs have not been produced, and states that as neither the specification nor the art available at the time of filing describe making stably transfected EGs and using these to make transgenic avians, undue experimentation would have been required by one skilled in the art to determine how to use the transfected EGs of the claimed invention.

The Office Action further states that the only disclosed use for the claimed transfected EGs is to make transgenic birds that have an altered phenotype or produce heterologous protein that can be recovered. Wall (1996) is cited as describing a number of factors that make it impossible to predict the effect that expression of an exogenous gene in a transgenic avian will have on the phenotype of the transgenic bird. For example, the position in the genome at which the transgene inserts, the interaction of host proteins that regulate gene expression with regulatory nucleic acid sequences in the inserted DNA, and the flanking host chromosomal DNA, all contribute to the unpredictability of both the level of expression, and the tissue-specific pattern of the expression of a transgene in a transgenic avian. The Office Action states that because of the unpredictability of the expression of a transgene in a transgenic avian, one skilled in the art would have had to perform undue experimentation in order to use transfected EGs. See pages 5-6 of the Office Action.

The Applicants submit that the specification describes the claimed invention in such terms and in sufficient detail that at the time the application was filed, a skilled individual would have been able to follow the teachings of the specification to make transfected EGs according to the claimed method without undue experimentation. As discussed below Neither the recognized unpredictability of transgene expression in a transgenic avian, nor the recognized difficulty of stably transfecting avian cells with plasmid DNA expression constructs, would have made it necessary for one of skill in the art to perform undue experimentation in order to make or use the claimed invention. Furthermore, as discussed below, one skilled in the art at the time the invention was filed would also have known how to use the claimed transfected EGs to make transgenic, chimeric avian embryos and transgenic avians without undue experimentation. Therefore, the rejection of the claims as being non-enabled is improper and should be withdrawn.

The rejected claims are drawn to a method of making transfected avian EGs derived from PGCs that are cultured in vitro for a prolonged period (i.e., at least 14 days) in the absence of feeder cells according to the disclosed invention. Key to the claimed invention is the disclosed method for culturing avian PGCs in vitro for a prolonged period in the absence of feeder cells. Until the present invention, it was believed that long-term culture of avian PGCs in vitro required growing the PGCs on a feeder cell layer (for example, see Pain et al., 1996, page 2345).

Prior to the present invention, those skilled in the art were familiar with methods for transfecting PGCs, and inserting the transfected PGCs into avian embryos to produce transgenic, chimeric avian embryos and transgenic avians. For example, Vick et al. (Proc. R. Soc. Lond., 1993, 251:179-182, copy attached) described using a retroviral vector to stably transfect avian PGCs, and injecting the transfected PGCs into recipient embryos to produce transgenic birds, as discussed in the Applicants' reply to the first Office Action. Although Vick et al. did not report that they detected expression of the transgene in transgenic avians, they taught that studying the transgenic, chimeric birds produced by their methods can provide information about their reproductive biology, because the retroviral DNA inserts in an apparently random fashion, so that it is possible to distinguish individual transfected PGCs from each other in the gonad of a transgenic avian by the site of insertion of the foreign DNA (see page 182). Thus, one skilled in the art would have recognized that transgenic, chimeric avians such as those disclosed by Vick et al. are useful even in the absence of transgene expression.

Moreover, given the advanced state of the art of genetic engineering at the time the application was filed, one of skill in the art would reasonably have regarded the report by Vick et al. of using retrovirally transfected avian PGCs to produce a transgenic avian with 4.5 kilobases of stably integrated foreign DNA as strong evidence undue experimentation would

not have been required to practice the claimed method by making and using transfected EGs to produce transgenic embryos and avians in which a transgene is successfully expressed. The Vick et al. reference was the first report of the use of transfected PGCs to produce a transgenic avian. As stated by the authors on page 181, the aim of their work was “to demonstrate the feasibility of using primordial germ cells as a means of gaining access into the avian genome.” Prior to publication of the Vick et al. reference, Bosselman et al. (J. Reprod. Fert., 1990, 15E:200; see also, U.S. Patent No. 5,162,215) and Lee et al. (Proc. 4th World Cong. Genet. Livestock Prod., 1990, 16:107-110) had already demonstrated successful expression and inheritance of retrovirally introduced transgenes in transgenic avians, as described on pages 118-119 of Simkiss (1994, pages 106-137). In addition, Chen et al. (J. Reprod. Fertil. Suppl. 41:173-182, abstract attached) reported in 1990 that a retrovirally introduced transgene encoding bovine growth hormone was successfully expressed in transgenic chickens that grew larger and matured more rapidly than wild type birds. Accordingly, those skilled in the art understood at the time the application was filed that a transgene introduced by a retroviral vector into cells of a transgenic avian could be stably integrated into the avian genome and expressed at sufficient levels as to affect the phenotype of the host avian.

In describing the work of Vick et al., Simkiss states (pages 121-122):

“... It is clear, therefore, that PGCs can be used as vehicles for introducing foreign DNA into the genome of the bird, and subsequent experiments have been directed at characterizing this phenomenon and increasing its efficiency.

The protocol for producing transgenics by such a procedure would, in outline, consist of (a) isolating PGCs, (b) integrating foreign DNA into their genome, (c) screening the cells for suitable gene expression, (d) introducing these genetically manipulated cells into a recipient embryo to form a chimera and (e)

breeding from this chimera and selecting those offspring that contained the introduced genes.”

Following publication of Vick et al., Allioli et al. (1994) reported successful expression of the lacZ reporter gene in isolated avian PGCs cultured and retrovirally-transfected in vitro, as noted in Applicant’s previous response. By the time the present application was filed, retroviral vectors were used routinely for obtaining expression of transgenes in avian primary cell cultures and in embryonic chick tissues in ovo (see Bell et al., Mol. Biotechnol., 1997, 7(3)289-298, abstract attached). The characterization of the results reported by Vick et al. in the Simkiss (1994) review article described above, and reports of stable integration and successful expression of retrovirally introduced transgenes in transgenic avian cells in vitro and in vivo provide clear evidence that one skilled in the art at the time the application was filed would have regarded the Vick et al. references as showing that transgenic PGCs could be used to produce stably transfected transgenic avians in which transgene expression was reasonably expected to occur.

The Office Action states that the specification fails to enable one skilled in the art to use the claimed invention because the levels and patterns of expression of transgenes introduced into transgenic avians by the claimed invention are unpredictable. The Applicants respectfully traverse this ground of rejection, because it imposes limitations that are not recited in the claims. The specification states that objects of the invention are “to introduce desired nucleic acid sequences into avian embryonic germ cells which have been obtained by culture of PGCs for prolonged periods in tissue culture,” and to use transfected EG cells “for the production of transgenic, chimeric avians” (page 7, lines 2-13). The specification is directed at those skilled in the art, who recognized the significance of the inventor’s discovery and knew how to use it. The rejected claims recite a method for producing

transfected avian EGs that encompasses making EGs in which there is very little or even no detectable expression of the transgene. As taught by Vick et al. and as discussed above, one skilled in the art would have known that stably transfected avian EGs could be used to study avian reproductive biology, e.g., by using the inserted DNA to identify individual transgenic cells and their lineages in a chimeric avian, even when expression of the transgene is undetectable. Moreover, as discussed further below, at the time the application was filed, those skilled in the art recognized that it was not necessary to be able to predict the level or pattern of transgene expression, or the change in phenotype caused by expression of a transgene in a transgenic avian, in order to find utility in the transgenic avian or in the method and cells by which it is generated.

The Office Action asserts that the claimed methods are not enabled, because introduction of plasmid DNA expression constructs into avian PGCs or EGs is expected to result in transient transfection. At the time the application was filed, the difficulties associated with producing transgenic avians that are discussed in the Office Action were generally recognized by those skilled in the art. Simkiss (1994) taught that “during the development of procedures using PGCs it is most convenient to use vectors that are likely to give the highest rates of integration and at the present time these are retroviruses,” and that as of 1992, stable transfection of avian cells had only been achieved with retroviral vectors (pp. 129-130). In 1994, Love et al. reported making stably transfected transgenic chickens by microinjecting plasmid DNA into avian embryos (Biotechnology, 12(1):60-63, abstract attached). However, as discussed below, most attempts to introduce non-retroviral DNA into avian cells appear to have produced transiently transfected cells, and at the time the application was filed, methods comprising transiently transfecting avian cells, including PGCs, and inserting the transfected cells into recipient embryos to generate transgenic avians in which the transgenes are expressed, were widely used by those skilled in the art. They

recognized that transgenic avians in which transgenes are expressed, even if only transiently, are highly useful for studying the tissue-specific and developmental stage-specific properties of promoters and other nucleic acid sequences that regulate gene expression, and for studying the relationship between the level or pattern of transgene expression in tissues of a transgenic avian embryo or chick and any changes in phenotype caused by transgene expression. For example:

- Wagner et al. (Clin. and Exper. Hypertension, 1995, 17(4):593-605, copy attached) described using transgenic animals to analyze promoter regulatory elements for tissue-specific expression, and to identify cis-acting factors that control gene transcription (p. 594).
- Simkiss (1994) taught that the avian embryo is “probably the best-studied example of vertebrate development” (p. 107), and described a study by Perry et al. (1991) of the temporal and spatial expression of the lacZ gene in avian embryonic cells following microinjection of cloned reporter constructs into the germinal disc of a freshly fertilized avian egg (see pages 114-115).
- Wall (1996) taught that, in view of the unpredictability of transgene expression, the best way to test the operability of a DNA expression construct in a tissue of a transgenic animal is to introduce the construct into an animal of the species of interest and assay its expression in the target tissue in vivo (p. 62).
- Naito et al. (VI International Symposium on Avian Endocrinology, March 31-April 5, 1996, abstract attached) reported transfecting plasmid DNA containing a lacZ gene into cultured avian PGCs in vitro, and inserting the transfected PGCs into recipient embryos to produce transgenic, chimeric avian embryos with gonads in which there were PGCs in which the lacZ gene was expressed, and they stated that their method could be used to test the expression of exogenous DNA in the gonads of developing chick embryos.

- Demenix et al. (Biotechniques, 1994, 16(3):496-501, abstract attached) described transfecting plasmid DNA expression constructs encoding reporter genes under control of various promoters into chick embryos in vivo and measuring the tissue-specificity and temporal aspects of the level of transgene expression in cells of the developing embryo.
- Kelder et al. (Gene, 1989, 76(1):75-80, abstract attached) transfected a plasmid DNA expression construct encoding a reporter gene under control of an inducible promoter into cultured avian cells and measured the dependence of transgene expression on the concentration of inducer in the cell culture medium.
- Rosenblum et al. (Transgenic res., 1995, 4(3):192-198, abstract attached) used cationic liposomes to transfect plasmid DNA expression constructs encoding reporter genes under control of a RSV promoter into avian embryonic cells, they measured changes in the level of transgene expression in the transfected embryos over time, and reported that while stable integration of transfected DNA appeared to be a rare event, liposome-mediated transfection of embryos is useful for studying promoter activity in vivo and may be useful for transfecting genes to study embryonic development.
- Ono et al. (Exp. Anim., 1995, 44(4):275-278, abstract attached) injected cationic liposomes complexed with plasmid DNA expression constructs into avian embryos and transfected the PGCs in vivo, as evidenced by detection of transgene expression in the gonads at later embryonic stages.
- In similar experiments, Watanabe et al. (Mol. Reprod. Dev., 1994, 38(3):268-274, abstract attached) injected avian embryos with cationic liposomes complexed with plasmid DNA expression constructs encoding a lacZ reporter gene under control of either a RSV promoter or a chicken beta-actin promoter, obtained transfection of avian PGCs and other cell types in vivo, and then measured and compared the levels of transgene expression directed by the two

different promoters in the transfected tissues of the developing embryos, including in transfected PGCs that migrated to the gonads of the developing embryos.

The Federal Circuit has long held that 35 U.S.C. §112 does not require a specific disclosure of that which is already known to one of ordinary skill in the art. Case v. CPC International, Inc., 221 USPQ 196, 201 (Fed. Cir. 1984). The claimed invention corresponds closely to the stated objects of the invention, “to introduce desired nucleic acid sequences into avian embryonic germ cells which have been obtained by culture of PGCs for prolonged periods in tissue culture,” and to use such transfected EG cells “for the production of transgenic, chimeric avians.” The published scientific articles described above show that at the time the application was filed, one skilled in the art would have known how to use the claimed methods for producing avian EGs that are transfected either transiently or stably with a DNA expression vector, and how to use the transfected EGs to make transgenic, chimeric avian embryos and transgenic avians. For example, one skilled in the art would know that transgenic, chimeric avian embryos and transgenic avians produced by the claimed invention are useful for studying tissue-specific promoter activity and the effects of transgene expression in the developing avian embryo, as discussed above.

The present invention facilitates methods for transfecting avians that were long-recognized as being needed by those skilled in the art. Simkiss (1994) discussed the feasibility of transfecting isolated avian PGCs in vitro and using the transfected PGCs to make transgenic avians, and the desirability of methods that would facilitate this approach; for example, to permit the screening of transfected PGCs in vitro for suitable gene expression (see pages 121-122). Simkiss stated that at the time of writing (1992), PGCs could only be cultured for short periods, and that a method permitting long-term culture of PGCs in vitro was needed to facilitate genetic manipulation of PGCs for production of transgenic, germ line chimeras (p. 124). Vick and Simkiss stated on page 181, column 2, that “clearly it is the

number of primordial germ cells that can be obtained rather than their cellular maturity that determine[d] the success rate in forming these [transgenic] embryos.” Vick and Simkiss obtained a higher rate of success using PGCs from the germinal crest before their vascular migration because they were able to isolate more PGCs. Simkiss (1994) also discusses the possibility that a method for long-term culturing of PGCs might give rise to embryonic germ cells that could be used to produce chimeric germ-line avians (page 123). As Applicants discussed in their replies to the earlier Office Actions, one skilled in the art would reasonably have expected that the disclosed methods for long-term culture of avian PGCs would make it easier to practice the methods for transfecting and using PGCs described in the prior art. Using the present invention, Vick et al. could have obtained as many cells as they needed, and because the present invention allows for long term culturing of cells, it would have permitted screening the transfected PGCs prior to chimera production, as suggested by Simkiss. Moreover, since the present method generates EG cells, one skilled in the art would have recognized that methods such as those used by Vick and Simkiss, in combination with the disclosed methods for producing pluripotent EG cells, could be used to produce transgenic chimeric somatic and germ-line avians, as speculated by Simkiss.

Those skilled in the art at the time the application was filed recognized that transiently transfected PGCs and EGs could be used to make transgenic, chimeric embryos and chicks that can be studied to determine tissue-specific and developmental stage-specific activities of nucleotide sequences that regulate gene expression, and to determine the effects of transgene expression on avian development, as discussed above. The level of skill in the art is high. Scientific articles such as Simkiss et al. expressly described a need for the development of methods such as the claimed method that permits long-term culture of PGCs in vitro in order to facilitate the genetic manipulation of PGCs and EGs for production of transgenic, somatic and germ line chimeras, also discussed above. Accordingly, the Applicants submit that at the

time the application was filed, one skilled in the art would have been able to follow the teachings of the specification and practice the claimed methods for producing transfected avian EGs and for using these to produce transgenic, chimeric avian embryos and transgenic avians, without having to perform undue experimentation. The Applicants therefore respectfully request reconsideration and withdrawal of the rejections under 35 U.S.C. §112, first paragraph.

The Applicants greatly appreciate the Examiner's indication of the scope of claims that he regards as being enabled by the description.

Regarding Rejection of the Claims Under 35 U.S.C. §112, Second Paragraph:

The claims have been amended to remove the language that provided the grounds for rejection under 35 U.S.C. §112, second paragraph, set forth in the Office Action.

Reconsideration and withdrawal of the rejections under §112, second paragraph is respectfully requested.

Regarding Rejection of Product Claims 21 and 22 Under 35 U.S.C. §102:

Claims 21 and 22, rejected under 35 U.S.C. §102(b) and 102(e), were amended in the reply filed June 3, 2002, so that they are no longer drawn to an avian EG cell line.

Accordingly, reconsideration and withdrawal of the rejections of claims 21 and 22 under 35 USC 102 is respectfully requested. The Applicants reserve the right to pursue claims to the products of the claimed invention in a different application.

Regarding Rejection of the Claims Under 35 U.S.C. §102 and 103 in view of Pain et al., alone or with Simkiss:

Claims 1, 3-11, 14-16, and 20-22 were rejected under 35 U.S.C. §102(b) on the grounds that the claimed method for prolonged culture of avian PGCs resulting in production of EG cells is anticipated under 35 USC 102(b) by Pain et al. (1996), as supported by Simkiss (1994). The examiner asserts that the claimed method for culturing PGCs for prolonged periods is not distinct from the method for culturing avian stage X blastoderm cells in the absence of feeder cells described by Pain et al.

The Applicants submit that Pain et al. do not anticipate the claimed methods because they do not disclose or suggest the claimed method comprising culturing avian PGCs *in vitro* in medium containing LIF, bFGF, SCF, and IGF-1, for at least 14 days in the absence of feeder cells, nor do they disclose or suggest that prolonged culture of avian PGCs *in vitro* under such conditions will result in production of pluripotent EG cells. In fact, Pain et al. and other prior art references expressly teach away from long-term culture of avian PGCs in the absence of feeder cells.

The Pain et al. reference described evaluating the effects of various growth factors on cultured avian embryonic cells by culturing chicken and quail cells from dissociated stage X blastoderms *in vitro* in medium containing LIF, bFGF, SCF, and IGF-1 in the presence and absence of feeder cells for 3-5 days, and then scoring or counting the alkaline phosphatase-positive colonies (page 2341, lower right column; and Fig. 2 on page 2342). Pain et al. reported that under their conditions, the feeder cells “strongly promoted the development of alkaline phosphatase-positive colonies (page 2341, bottom of right column). Pain et al. also disclosed studying the importance of LIF in long-term culture of chicken embryonic cells (CECs) by culturing the CECs for 11 passages in medium (“ESA complete medium”) containing LIF, bFGF, SCF, and IGF-1, and then culturing the cells for an additional 24 days

in the same medium with or without LIF (page 2343, paragraph bridging columns, and Fig. 4). Pain et al. reported that LIF is required for the long-term growth of avian embryonic cells and the expression of antigens characteristic of ES cells, and that avian embryonic cells expressing antigens characteristic of ES cells could be maintained for at least 35 passages, i.e. more than 160 days, in the presence of LIF. The paragraph on page 2343 of Pain et al. describing the long-term culturing of CECs does not specify whether the long-term cultures of CECs in medium containing LIF, bFGF, SCF, and IGF-1 were carried out using feeder cells; however, Pain et al. clearly stated on page 2345 (right column) that their long-term culture conditions included the use of feeder cells:

“The culture conditions which included the use of mouse embryonic feeder cells and the inclusion of LIF, IL-11, SCF, bFGF, IGF-1, and ARMA in the medium, facilitated the proliferation of cells with an undifferentiated phenotype during more than 35 passages, i.e., more than 160 days.”

In summary, Pain et al. described culturing PGCs in vitro in medium containing LIF, bFGF, SCF, and IGF-1 in the presence and absence of feeder cells, they reported that the cells grew much better in the presence of feeder cells, and they taught that PGCs could be cultured for a prolonged period in medium containing LIF, bFGF, SCF, and IGF-1 in the presence of feeder cells. Nothing in Pain et al. or Simkiss suggests the claimed invention comprising culturing avian PGCs *in vitro* in medium containing LIF, bFGF, SCF, and IGF-1, for at least 14 days in the absence of feeder cells, and nor does either reference, alone or in combination, suggest that prolonged culture of avian PGCs *in vitro* under the claimed conditions will result in production of pluripotent avian EG cells. Accordingly, reconsideration and withdrawal of the rejection under 35 USC 102(b) is respectfully requested.

Claims 1 and 2 were rejected under 35 U.S.C. §103(a) on the grounds that the claimed method for prolonged culture of avian PGCs resulting in production of EG cells is anticipated under 35 USC 102(b) by Pain et al. (1996), as supported by Simkiss (1994), as set out in the previous Office Action. The previous Office Action stated that Pain et al. disclosed obtaining EG cells after long-term culturing of PGCs, as evidenced by production of germline and somatic cell chimeras after introduction of the into stage X chicken embryos. The Office Action asserted that the description by Pain et al. of short-term culture conditions used to test the effects of different combinations of growth factors would have motivated one of ordinary skill in the art to use the conditions of the claimed method in order “to optimize the conditions required to obtain EG/ES cells.”

The Applicants respectfully traverse the rejection of claims 1 and 2 under 35 U.S.C. §103(a) as set forth in the present and previous Office Actions. In the first place, the previous Office Action mischaracterizes the method described by Pain et al. as the prolonged culture of avian PGCs. Pain et al. cultured blastoderm cells from a Stage X embryo which, in view of Simkiss, would have been expected to include a small percentage of cells that were PGCs, and they reported obtaining a mixed, non-clonal culture of cells capable of contributing to ectoderm and, with low frequency, also capable of populating the germ line (page 2346, right column). In providing explanations for the low frequency of germline transmission that they observed with the cultured cells obtained by their method, Pain et al. noted that they were unable to maintain clonal growth of chicken embryo cells (CECs), and that germline precursor cells may actually have been preferentially lost under their culture conditions (page 2346, right column).

As discussed above, the only disclosure in the Pain et al. reference of culturing avian embryonic cells in the presence and absence of feeder cells was the description of a short-term in vitro method for evaluating the effects of various growth factors on cultured avian

embryonic cells, in which cells from dissociated stage X blastoderms were cultured in vitro in medium containing LIF, bFGF, SCF, and IGF-1 in the presence and absence of feeder cells for 3-5 days, and then scored for production of alkaline phosphatase-positive colonies (page 2341, lower right column; and Fig. 2 on page 2342). In describing the results they obtained from this method, Pain et al. actually taught away from the claimed invention, in reporting that feeder cells “strongly promoted the development of alkaline phosphatase-positive colonies (page 2341, bottom of right column), and that their long-term culture conditions included the use of feeder cells (see page 2345, right column). One of ordinary skill in the art would also have known that other research groups had found feeder cells to be necessary in long-term culturing of avian embryonic cells in vitro; for example, see Petite et al., U.S. Patent No. 5,340,740.

Thus, the cited prior art references did not provide any suggestion or motivation to one of ordinary skill in the art to practice the claimed method by culturing avian PGCs in vitro in medium containing LIF, bFGF, SCF, and IGF-1, for at least 14 days in the absence of feeder cells; nor did the prior art suggest culturing avian PGCs in vitro under the claimed conditions for a prolonged period to obtain pluripotent avian EG cells, with any expectation of success. Accordingly, the claimed methods would not have been obvious to one of ordinary skill in the art in view of Pain et al., taken with Simkiss, at the time the application was filed, and the Applicants respectfully request that the rejection under 35 U.S.C. §103(a) be reconsidered and withdrawn.

Regarding Rejection of the Claims for Double Patenting:

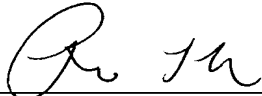
The Applicants acknowledge the obvious-type double patenting rejections set forth in the Office Action, and request that they be held in abeyance until the indication of allowable subject matter.

The issues raised by the Office Action dated December 2, 2001, have been addressed in this Reply, and the claims are now believed to be in form for allowance. If the Examiner has any further questions or issues to raise regarding the subject application, it is respectfully requested that he contact the undersigned so that such issues may be addressed expeditiously.

Respectfully submitted,
PILLSBURY WINTHROP LLP

Date: June 17, 2002

1600 Tysons Boulevard
McLean, VA 22102
(703) 905-2000 Telephone
(703) 905-2500 Facsimile

By: 
Robin L. Teskin
Registration No. 35,030

APPENDIX**IN THE SPECIFICATION:**

The paragraph beginning at line 3 of page 20 is amended as shown:

-- As discussed, a significant application of the subject PGCs, which can be maintained in culture for long periods, is for the production of transgenic chimeric avians. This will be accomplished by introducing a desired DNA sequence into the cultured PGCs. Means for introducing DNAs into recipient cells are known and include lipofection, transfection, microinjection, transformation, microprojectic techniques, etc. In particular, the present inventors initially elected to introduce a vector containing a reporter gene by lipofection. However, while transiently transfected PGCs were produced, a stable transfected cell line has not, as yet, been isolated. However, it is expected that this can be accomplished by known techniques using the subject PGCs. --

The paragraph beginning at line 17 of page 20 is amended as shown:

-- Preferably, a DNA will be introduced that comprises [encodes] a desired gene, e.g., a gene that encodes therapeutic polypeptide, growth factor, enzyme, etc., under the regulatory control of sequences operable in avians. Preferably, these regulatory sequences will be of eukaryotic origin, most preferably avian, e.g., chicken regulatory sequences. Promoters operable in avian cells, e.g., derived from avian genes or viruses are known in the art. --

IN THE CLAIMS:

Claims 13, 18, 23 and 26 are further amended as follows:

13. (Twice Amended) The method of Claim 12, wherein said nucleic acid comprises a nucleotide sequence that encodes a polypeptide and is [expressed in an egg] functionally linked to gene expression regulatory sequences that are operable in an avian cell.

18. (Twice Amended) The method of Claim 17, wherein said nucleic acid comprises a nucleotide sequence that encodes a polypeptide and is [expressed in an egg of an avian embryo having said nucleic acid sequence] functionally linked to gene expression regulatory sequences that are operable in an avian cell.

23. (Twice Amended) The method of Claim [1] 12, wherein said nucleic acid encodes a polypeptide that is a growth factor or an enzyme.

26. (Twice Amended) A method of producing germline [or] and somatic cell chimeric avians which comprises:

- (i) isolating primordial germ cells (PGCs) from a Stage XII-XIV avian embryo;
- (ii) maintaining such PGCs in a tissue culture medium containing at least the following growth factors:
 - (1) leukemia inhibitory factor (LIF),
 - (2) basic fibroblast growth factor (bFGF),
 - (3) stem cell factor (SCF) and
 - (4) insulin-like growth factor (IGF),for a sufficient time to produce embryonic germ (EG) cells;

- (iii) transferring said EGs into a recipient Stage X avian embryo of the same species as the avian used to obtain said isolated PGCs;
- (iv) allowing said recipient avian embryo to develop into a germline [or] and somatic cell chimeric avian having germline and somatic cells that have the genotype of said PGCs.

1: Biotechnology (N Y) 1994 Jan;12(1):60-3

Transgenic birds by DNA microinjection.

Love J, Gribbin C, Mather C, Sang H.

AFRC Roslin Institute Edinburgh, Roslin, Midlothian, U.K.

We have developed a method for production of transgenic chickens by DNA microinjection of chick zygotes followed by ex vivo embryo culture. The fate of plasmid DNA microinjected into the germinal disc of zygotes was analyzed in embryos which survived for at least 12 days in culture. Approximately half of the embryos contained plasmid DNA, 6% at a level equivalent to one copy per cell in all tissues analyzed. Seven chicks, 5.5% of the total number of injected ova, survived to sexual maturity. One of these, a cockerel, transmitted the exogenous DNA to 3.4% of his offspring. These G1 birds have reached sexual maturity and have been bred to produce transgenic offspring, demonstrating that stable transmission of foreign DNA can be obtained by our method.

PMID: 7764327 [PubMed - indexed for MEDLINE]

1: Development 1992 Feb;114(2):331-8

Distribution analysis of transferred donor cells in avian blastodermal chimeras.

Watanabe M, Kinutani M, Naito M, Ochi O, Takashima Y.

Department of Biology, Faculty of Science, Ehime University, Japan.

Blastodermal chimeras were constructed by transferring quail cells to chick blastoderm. Contribution of donor cells to host were histologically analyzed utilizing an in situ cell marker. Of the embryos produced by injection of stage XI-XIII quail cells into stage XI-2 chick blastoderm, more than 50 percent were definite chimeras. The restriction on the spatial arrangement of donor cells was induced by varying the stage of host. Ectodermal chimerism was limited to the head region and no mesodermal chimerism was shown when the quail cells were injected into stage XI-XIII blastoderm. Mesodermal and ectodermal chimerisms were limited to the trunk, not to the head region, when the quail cells were injected into the stage XIV-2 blastoderm. In these chimeras, however, some of the injected quail cells formed ectopic epidermal cysts. Consequently, the stage XIV-2 blastoderm may become intolerant of the injected cells. Our results suggest that it is possible to obtain chimeras that have chimerism limited to a particular germ layer and region by varying the stage of donor cell injection. Injected quail cells contributed to endodermal tissues and primordial germ cells regardless of the injection site. The quail-chick blastodermal chimeras could be useful in the production of a transgenic chicken and in the investigation of immunological tolerance.

PMID: 1591995 [PubMed - indexed for MEDLINE]

1: Mol Biotechnol 1997 Jun;7(3):289-98

Replication-competent retroviral vectors for expressing genes in avian cells in vitro and in vivo.

Bell EJ, Brickell PM.

Department of Developmental Neurobiology, UMDS, Guy's Hospital, London, UK.

Replication-competent retroviral vectors based on Rous sarcoma virus (RSV) are becoming increasingly popular for expressing genes in both primary cell cultures and embryonic chick tissues in ovo. In this article, we review the features of RSV and its life cycle that make it suitable for use as a vector. We describe the design and use of the RCAS and RCAS (BP) series of vectors, which are currently the most widely used RSV-based vectors, illustrating both their strengths and weakness. Finally, we outline laboratory protocols suitable for the banding of these retroviral vectors.

Publication Types:

Review

Review, Tutorial

PMID: 9219242 [PubMed - indexed for MEDLINE]

1: J Reprod Fertil Suppl 1990;41:173-82

Vectors, promoters, and expression of genes in chick embryos.

Chen HY, Garber EA, Mills E, Smith J, Kopchick JJ, DiLella AG, Smith RG.

Department of Growth Biochemistry & Physiology, Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065.

Transgenic chickens were produced by injecting the Day-1 egg with 10(5) infectious particles of a replication-competent virus based on the Schmidt-Ruppin A strain of Rous sarcoma virus. The chickens were resistant to transforming subgroup A virus containing the src gene but not the corresponding subgroup B virus. Transgenic chickens producing bovine growth hormone (bGH) were generated using a modified virus containing the Bryan high titre polymerase gene. The virus was constructed with the bGH gene and the mouse metallothionein promoter in the reverse orientation relative to the viral structural genes. Two male chickens produced serum concentrations of approximately 100 ng bGH/ml; the birds were larger than controls and matured more rapidly. Transgenic mice required for the analysis of skeletal muscle-specific expression in vivo were produced using 5'-flanking regions of the chicken alpha-skeletal actin promoter linked to a luciferase reporter gene to determine the region essential for tissue-specific expression. The defined promoter sequences are to be used in experiments designed to direct expression of growth-promoting genes in skeletal muscle of chickens.

PMID: 2170640 [PubMed - indexed for MEDLINE]

1: Dev Biol 1994 Sep;165(1):30-7

Use of retroviral vectors to introduce and express the beta-galactosidase marker gene in cultured chicken primordial germ cells.

Allioli N, Thomas JL, Chebloune Y, Nigon VM, Verdier G, Legras C.

Centre de Genetique Moleculaire et Cellulaire, CNRS UMR106, INRA LA810,
Universite Claude Bernard Lyon-I, Villeurbanne, France.

Three methods of isolating primordial germ cells (PGCs) from gonads of 5-day-old chick embryos were compared. PGCs were then cultured in vitro in DMEM/F12 medium containing 10% fetal calf serum. BrdU incorporation showed that at least 10% of the PGC population were dividing, under our culture conditions, during the 2nd day of in vitro culture. During this culture period, PGCs were exposed to avian leukosis sarcoma virus-based retroviral vector pseudotyped with subgroup A envelope, carrying the LacZ reporter gene. X-Gal staining showed that PGCs were permissive to infection, with more than 50% of PGCs expressing the beta-Gal protein. These data represent the first demonstration that PGCs, isolated from gonads of 5-day-old chick embryos, are able to divide in vitro and that it is possible to introduce and express exogenous DNA in chick PGCs maintained in vitro.

PMID: 8088448 [PubMed - indexed for MEDLINE]

1: Exp Anim 1995 Oct;44(4):275-8

Gene transfer into circulating primordial germ cells of quail embryos.

Ono T, Muto S, Matsumoto T, Mochii M, Eguchi G.

Faculty of Agriculture, Shinshu University, Ina, Japan.

During early stages in avian embryogenesis primordial germ cells (PGCs) show a unique migration pathway toward the gonadal anlage through the circulation. In the present study, liposomes consisting of plasmid DNA (pMiwZ; containing lacZ as a reporter) and Lipofectin were injected into the marginal veins of quail embryos during the stages PGCs were circulating in the blood vessels. The lacZ expression was then histochemically detected in the gonads at later embryonic stages, indicating the expression of the injected DNA in PGCs.

PMID: 8575540 [PubMed - indexed for MEDLINE]

1: Mol Reprod Dev 1994 Jul;38(3):268-74

Liposome-mediated DNA transfer into chicken primordial germ cells in vivo.

Watanabe M, Naito M, Sasaki E, Sakurai M, Kuwana T, Oishi T.

National Institute of Animal Industry, Ibaraki, Japan.

In embryogenesis, avian primordial germ cells (PGCs) circulate temporarily in the blood vessels at stages 10-15 (Hamburger and Hamilton, 1951), before reaching the gonads. In an attempt to transfer cloned genes into PGCs, liposome consisting of reporter plasmid DNA and N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammoniummethanesulfonate was injected into the marginal veins of embryos at stages 11-15. As reporter plasmids, pRSVZ and pAcZ harboring the Escherichia coli lacZ gene driven, respectively, by the Rous sarcoma virus (RSV) promoter and the chicken beta-actin gene promoter were used. First, 55 embryos were injected with liposome containing pRSVZ and stained for the bacterial beta-galactosidase activity 24 hr after injection. In all the embryos, cells positive for beta-galactosidase activity were observed among the blood cells, endothelial cells, and endocardium cells of the heart, suggesting that transfection took place within the circulatory system. Then, embryos were injected with liposome containing pRSVZ or pAcZ, and stained 2 or 3 d after injection. PGCs positive for beta-galactosidase activity were observed in the gonads in four out of 44 embryos injected with pRSVZ, and 29 out of 71 embryos injected with pAcZ, indicating that the plasmid DNA was transferred into PGCs developing normally. The average number of positive PGCs per embryo was 0.2 and 2.1, respectively, when pRSVZ and pAcZ were introduced. The difference in the number of positive PGCs detected after introduction of the two plasmids suggests that the actin promoter has a higher level of transcriptional activity in PGCs than does the RSV promoter.

PMID: 7917278 [PubMed - indexed for MEDLINE]

1: Transgenic Res 1995 May;4(3):192-8

In ovo transfection of chicken embryos using cationic liposomes.

Rosenblum CI, Chen HY.

Department of Genetics and Molecular Biology, Merck Research Laboratories,
Rahway, NJ 07065, USA.

It is reported that cationic liposomes are capable of transfecting embryos in unincubated fertile chicken eggs and that the cationic liposome, TransfectAce, has superior properties to Lipofectin. In order to determine the duration of expression of genes introduced in this way, embryos were transfected with an expression vector encoding the firefly luciferase cDNA under the control of the Rous sarcoma virus long terminal repeat (LTR). Luciferase activity could be observed consistently in day 3 embryos and activity was detectable up to day 8 of incubation. The relative expression of luciferase under the control of different viral promoters was compared in transfected chicken embryo fibroblasts and day 3 embryos. The cytomegalovirus immediate early promoter and the SV40 early promoter directed the highest amount of expression in fibroblasts while the Rous sarcoma virus LTR caused the highest amount of expression in embryos. Chicken embryo fibroblasts were transfected with the luciferase vector in order to examine duration of reporter gene expression in vitro. Luciferase expression was decreased exponentially over a 24-day period after which point luciferase activity could no longer be detected. These data suggest that stable integration of transfected DNA using liposomes is a rare event. Nevertheless, liposome-mediated transfection of embryos is suitable for the examination of promoter activity in vivo and may be a useful method to transfect genes to study embryonic development.

PMID: 7795662 [PubMed - indexed for MEDLINE]

1: Gene 1989 Mar 15;76(1):75-80

Activation of the mouse metallothionein-I promoter in transiently transfected avian cells.

Kelder B, Chen H, Kopchick JJ.

Department of Zoological and Biomedical Sciences, Ohio University, Athens 45701.

The induction of the mouse metallothionein-I (MT-I) transcriptional regulatory region by heavy metals in cultured avian cells was studied in a transient-expression assay system for a growth hormone. The MT-I promoter was shown to be inducible by 10 microM to 90 microM ZnCl₂ in a dose-dependent manner.

PMID: 2744481 [PubMed - indexed for MEDLINE]

1: Biotechniques 1994 Mar;16(3):496-501

Temporal and spatial expression of lipospermine-compact genes transferred into chick embryos in vivo.

Demeneix BA, Abdel-Taweb H, Benoist C, Seugnet I, Behr JP.

Museum Nationale d'Histoire Naturelle, Paris, France.

We have optimized a lipospermine-based transfection method for introducing genes into intact vertebrate embryos in vivo. The method employs small amounts of the cationic lipid Transfectam (DOGS), in a concentrated (40 mM) ethanolic solution, to compact and to transfer exogenous genes into chick embryos during the early stages of development (< 36 h of incubation). Plasmid vectors containing the reporter gene luciferase were used to follow the time course of expression. Luciferase activity was detected as early as 12 h post-transfection and was highest at this time. Enzyme activity then decreased over the next two days and was usually undetectable by 72-h post-transfection. To follow the spatial expression of the exogenous genes, a Rous sarcoma virus (RSV)-beta-galactosidase vector was used. When the transfection complex was applied externally around the developing embryo, the main site of expression was the cardiac tissue. Expression could be targeted to the nervous system by micro-injecting the DNA/DOGS (DNA/dioctadecylamidoglycylspermine) complex into the developing brain. The results show that reporter genes can be efficiently expressed in both the developing central nervous system and heart. This raises the possibility that lipospermines can be used to transfer functional genes into embryos during defined periods of development and also to deliver genes in other species and in other in vivo contexts.

PMID: 8185924 [PubMed - indexed for MEDLINE]

{Home} . [Biology] . [Chemistry] . [Environmental] . [Materials] . [Science Progress]
Science & Technology Letters

Science Reviews

Abstracts from

VI International Symposium on Avian Endocrinology

March 31 - April 5, 1996 Chateau Lake Louise, Alberta

Continued

Sex Determination in Birds from the Perspective of Studies on Triploid Intersexes

B.L. Sheldon¹, M.H. Thorne^{1,2}

1CSIRO Division of Animal Production, Locked Bag 1, Delivery Centre, Blacktown, NSW 2148 Australia; 2Current Address: 41 Roma Rd, St. Ives, NSW 2075 Australia
(FAX: 6128402940)

Recent advances in knowledge of sex determination in mammals have depended largely on analysis of individuals with aberrant sex chromosome constitution, such as XO or XY females and XX or XXY males. Analogous individuals, ZZ females and ZW males, have not been detected in chickens. The development of a unique "triploidy" line of chickens has enabled the study of large numbers of triploid female intersex chickens (3A.ZZW), and a few associated chimeric individuals. These studies are reviewed in this paper. They demonstrate that (i) masculinization of the external phenotype of 3A.ZZW apparent females becomes obvious only from about sexual maturity, (ii) initial determination and differentiation of 3A.ZZW embryos as female is normal and complete, (iii) masculinization of the left ovary and development of a right testis in place of a regressed right ovary already starts before hatching, (iv) this masculinization of the gonads is reversible (at least temporarily) by injection of estrogen at 96 h of incubation, (v) the sex phenotype of the chimeras is dependent primarily on the presence of a W chromosome, but affected by the proportions of diploid and triploid cells and the dosage of Z and W chromosomes. The results support the hypothesis that there is a female (ovary) determining gene on the W chromosome, the effect of which is able to be inhibited or partly reversed by more than one dose of male (testis) determining gene(s) on the Z chromosome.

Expression of Genes Involved in Estrogen Synthesis and Expression of the Estrogen Receptor Gene during Early Gonadal Development of Chickens

S. Mizuno, O. Nakabayashi, H. Kikuchi¹, T. Kikuchi¹

T. Maeda

Faculty of Applied Biological Science, Hiroshima University, Higashi-Hiroshima, 739 Japan
(FAX: 81-824-22-7067; E-mail: temaeda@ue.ipc.hiroshima-u.ac.jp)

To clarify the divisional ability of primordial germ cells (PGCs), the quantity of DNA and frequency of the final stage of metaphase in PGCs at stages 14-15 (before settling in the germinal ridge) and stages 17-20 (after settling in the germinal ridge) were analyzed using an interactive laser cytometer.

When the quantity of DNA was graphically presented, two peaks were recognized in all stages. The value of second peak was approximately twice that of the first. The PGCs having two nuclei in one cell were recognized in each stage and were considered to be the final stage of metaphase in cell division. The amount of DNA in metaphase correlated with that in the second peak. The frequency of final stage metaphase in PGCs after settling in the germinal ridge was markedly increased comparing with that before, and significantly increased with advancing embryonic stage.

From these results, it seems that intense proliferation of chicken PGCs occurs after settling in the germinal ridge.

Expression of Exogenous DNA in Embryonic Gonads by Transferring Primordial Germ Cells Transfected *in Vitro*

M. Naito, a. Tajima¹, Y. Yasuda², M. Sakurai, T. Kuwana³

National Institute of Animal Health, Tsukuba 305, 1Tsukuba University, Tsukuba, 305,
2Kumamoto University, Kumamoto 860, 3National Institute of Minamata Disease, Kumamoto
867, Japan

*(FAX: 81-298-38-7880; E-mail: mnaito@niah.affrc.go.jp)

Gene transfer into primordial germ cells (PGCs) and the production of viable offspring from germline chimeric chickens provides a powerful tool for studying avian endocrinology. PGCs collected from the embryonic blood of White Leghorns (WL) were concentrated. Transfection of PGCs was achieved using a cationic lipid (DOTAP, Boehringer Mannheim Biochemica). The PGCs were incubated with DOTAP:DNA (pAcZ, *lacZ* gene under the control of chicken β -actin gene promoter) mixture for 5 hours at 38°C, washed with culture medium and 300 PGCs were injected into the blood stream of recipient embryos from which blood had been drawn prior to the injection. The recipient embryos were incubated for 3 days and *lacZ* gene expression was detected in the gonads by X-gal staining. The percentage of embryos which expressed the *lacZ* gene in the left and/or right gonads was 71.2% (37/52), and the number of positive cells for the *lacZ* gene in the gonads was variable; a few to more than 50 cells. Thus, the *lacZ* gene was successfully introduced into the PGCs *in vitro* and was expressed in the gonads of recipient embryos. This technique provides an experimental system for testing the expression of exogenous DNA in the gonads of developing chick embryos, and also contributes to the production of transgenic chickens.

CLIN. AND EXPER. HYPERTENSION, 17(4), 593-605 (1995)

REVIEW

TRANSGENIC ANIMALS AS MODELS FOR HUMAN DISEASE

Juergen Wagner, Felix Thiele, Detlev Ganten

German Institute for High Blood Pressure Research and Max Delbrück Center for Molecular
Medicine, Berlin, Germany

Keywords: Transgenic animals, human disease, genetics, cardiovascular disease, oncology,
immunology, toxicology, Alzheimer's disease, embryo development

Abstract

Since its first description in 1981 (1), transgenic technology has greatly influenced the focus and direction pace of biomedical research. Introduction of foreign DNA into the genome of animals by microinjection into fertilized oocytes is now used in almost every field of research spanning from oncology, immunology and neurology to cardiovascular medicine. The ability

Address for correspondence:

Dr. Juergen Wagner
Max Delbrück Center for Molecular Medicine
Franz-Gross-Haus/134D
Wiltbergstraße 50
13122 Berlin
Germany

to integrate genes in the germline and their successful expression in the host provides an opportunity to study the role of a certain gene in the initiation and propagation of disease. Transgenic methodology serves as the link between molecular biology, introducing *in vitro* a defined genetic modification and whole animal physiology, with the resulting *in vivo* alteration of body function. This potential has been exploited to study the pathophysiological role of human genes. Transgenic animals have been used to study aspects of tumor development, immune regulation, cardiovascular development and atherosclerosis. These studies have provided new insights into the genetic origin of certain diseases and have improved our understanding of pathological processes on the cellular level. As a future goal, these studies may also serve the development of new diagnostic tools or novel therapeutic strategies such as gene therapy.

Introduction

Our understanding of body function in health and disease has been advanced in the past primarily by the use of animal models for human disease. However, to establish a relationship between the regulation of a certain gene and a complex disease process has been difficult. The expression of a foreign gene creates a defined genetic defect, which allows to closely correlate the effect of this gene to a physiological trait.

Studies on the regulation of gene expression and gene function in humans are strongly limited due to ethical reasons. Expression of human transgenes in animals therefore is an elegant way to obviate these difficulties. Experiments using transgenic animals can be divided into four categories: 1) Studies on gene regulation: which include the expression of only the regulatory elements of a gene such as the promoter region in transgenic animals. The promoter region of a gene is connected to a reporter gene which is easily detectable. Such studies using the promoter region are of use in the analysis the regulatory elements for tissue-specific expression and identification of cis-acting factors controlling gene transcription. 2) Investigation of the function of a gene product: Here, the gene of interest is under control of either the natural homologous promoter or a heterologous one which directs expression to specific cells and tissues. Additionally, mutants of the gene of interest may be introduced for

analysis of specific biochemical properties. 3) *In vivo* immortalization of cells: this is accomplished e.g. by fusion of a gene promoter region to a SV 40 large T antigen. The immortalized cell lines are then isolated for *in vitro* analysis. Finally 4) expression of proteins in mammary gland tissue in order to obtain large quantities of secretory protein within the milk.

Transgenic technology was originally developed in the mouse and, therefore, most transgenic studies are performed in this species. The mouse model provides several advantages such as comparatively numerous offspring with short generation times and well-known genetics. For specific research questions, however, other species have been used such as rat, sheep, rabbit, goat and zebrafish (2-5). In cardiovascular research rats have been the animals of choice to study cardiovascular function. They are suitable for pharmacological tests and have provided animal models for hypertension, cardiac and renal disease.

The most widely used technique applied for transgenic production is micromanipulation of fertilised oocytes from superovulated donor animals and microinjection of DNA into the pronucleus (1,6). The DNA-injected eggs are reimplanted into pseudopregnant foster mothers and the offspring are then analysed for the presence of the foreign DNA in the genome. This technique is basically common to all animal species where this technology has been applied. Time schedule, hormone treatment and operating procedure, however, require adjustment to the respective species. Whereas microinjection aims at implantation of foreign genomic material into the germline, different techniques have been developed to insert DNA into *somatic* cells (7-10). This implies transgenesis in cells which are not part of the germline and, therefore, a genetic alteration can not be propagated to the offspring. These techniques often aim at gene therapeutic approaches where a defective gene can be replaced in function at some time in the ontogeny.

Three methodological approaches have been used to generate transgenic animals: i) injection of DNA into the pronucleus of fertilized oocytes as described above. ii) homologous recombination in embryonic stem cells of mice (11) and iii) retroviral infection of preimplantative blastocysts (12). At present, research efforts are focused on the first two techniques which represent two sides of a coin. The "knock out" approach achieved with homologous recombination yields a "loss-of-function" study, in contrast to the "gain-of-

function" approach which is used in microinjection causing overexpression of a gene. "Loss-of-function" is a tool to analyse the function of a gene by functional interruption and is achieved by inserting a disruptive sequence *in vitro*. The endogenous gene is replaced with the mutated gene by homologous recombination in the stem cell. Despite the precision of this method, functional conclusions are not easily drawn from such experiments. For example, gene "knock-outs", especially of transcription factors, have often demonstrated no apparent phenotype due to the functional redundancy of cell and gene regulatory systems. Other gene disruptions have precluded extensive analysis due to embryonic lethality. Studies using partial "loss-of-function"-mutants or double knock outs are under way to obviate these difficulties (13). Thus far, embryonic stem cell technology has only been successful in mice, however, the need to generate rat embryonic stem cells has been recognised for physiological and pharmacological investigations.

Homologous recombination allows replacement of a the native gene by a mutant which can be analysed in the natural chromosomal environment, which affects gene expression and regulation. In contrast, transgenesis by micorinjection occurs by random insertion into the genome without control of copy numbers of DNA integrated. To achieve a reproducible gene integration, locus control regions or matrix attachment regions have been used as control elements to direct transgene integration (14-17). Another successful strategy has been not to inject the transgene alone but rather large DNA constructs such as yeast artificial chromosomes (18-19), in order to control the "environment" of the transgene. These techniques will in the future redefine the transgenic methodology and possibly other newly developed strategies for somatic transgenesis. Progress in this field can not be separated from gene therapeutic approaches, where a foreign gene will be transferred into somatic cells. A vast number of different *in vitro* and *in vivo* strategies exists for gene transfer. *In vitro* strategies use host cells that are isolated from the body, grown, stably transfected with a transgene and then reimplanted (10). *In vivo* studies directly apply DNA to the host either by direct injection into the tissue of interest, by liposomal transfection, by retroviral or adenoviral infection (8,9) or by receptor-mediated uptake e.g. by via the transferrin receptor. A detailed description of these technologies, however, is beyond the scope of this article.

Transgenic animals in cardiovascular disease

The cardiovascular system has been the focus of interest for transgenic research due to the high cardiovascular morbidity and mortality in industrialized societies. Transgenic animals have been generated for almost every aspect of cardiovascular research from hypertension to formation of myocardial tumors. The candidate gene approach has been used to study the effects of gene products of hormones which are known to be involved in blood pressure regulation and which are supposed to play a role in the pathogenesis of hypertension. Other risk factors of cardiovascular disease such as atherosclerosis or hemostatic mechanisms have been investigated by transgenic techniques.

Candidate gene approach/neurohormonal studies

The regulation of cardiovascular function is complex and depends on many factors which interact in a defined spatial and temporal pattern. It is therefore difficult to assign a particular phenotype or functional parameter to a certain gene. Transgenic introduction of a gene into an organism does allow to define the contribution of a certain gene to the physiology or pathophysiology of cardiovascular function. Due to the multitude of hormones, regulatory peptides, cell signalling pathways etc., research has focused on the role of candidate genes. These are genes, which are known to be involved in cardiovascular regulation and, therefore, likely to play a role in dysfunction of the heart or the vascular wall as in hypertension. Since the expression of the transgene in animals is the only difference to transgene-negative control animals, a change in cardiovascular function can be correlated to the presence of the transgene.

The precursor of *arginine vasopressin*, *preproarginine vasopressin*, which is under control of the metallothionein promoter has been expressed in transgenic mice resulting in chronically elevated levels of vasopressin in the plasma (20). Increased levels of vasopressin were present in the plasma elevating serum osmolality to levels corresponding to mild nephrogenic diabetes insipidus. *Atrial natriuretic peptide* which is known to reduce blood pressure and to induce a

marked natriuresis has been expressed in mice to study the effects of chronically elevated ANP levels on cardiovascular function. Use of the heterologous mouse promoter transthyretin resulted in a ten-fold elevation of immunoreactive plasma ANP and significantly lowered blood pressure without altering plasma electrolyte balance (21).

Transgenic animals have also been generated in hypertension research. Besides the known influence of environmental factors on the development of high blood pressure, hypertension has a strong genetic background (22,23). Therefore, candidate genes of hypertension such as the components of the *renin-angiotensin system* have been studied in detail. This system is a major regulator of blood pressure and of sodium- and volume homeostasis. Renin genes of different species, as well as its substrate angiotensinogen, have been introduced into transgenic mice (24-27). Transgenic mice expressing both the rat or human renin and angiotensinogen gene developed elevated blood pressure levels (27,28). However, rats, as opposed to mice, have attracted much interest in the field of research, since they are more suitable for hemodynamic, pharmacological and functional studies. Rats with hereditary hypertension, such as spontaneously hypertensive rats, have been used as a model for primary human hypertension. Expression of the mouse renin-2-gene in transgenic rats has led to fulminant hypertension with values in the range of 220 mmHg systolic in heterozygous animals (3). Unexpectedly, despite the presence of an additional renin gene, these rats exhibit a low plasma renin activity, corresponding to low renin hypertension syndromes in humans. Transgenic rats with the human renin and angiotensinogen gene have also been generated which maintain the species-specificity of the human renin-substrate reaction (4).

Cardiovascular aspects can also be demonstrated in transgenic mice overexpressing *growth hormone* (29,30). Excess growth hormone in humans causes acromegaly and gigantism. Patients suffering from this disease frequently develop hypertension, although growth hormone by itself is not hypertensinogenic. Overexpression of a metallothionein-fusion gene in mice did not significantly raise blood pressure, but the vascular wall-to-lumen ratio was significantly altered in mesenteric arteries. The increase in wall thickness in these arteries may elevate peripheral resistance and thus contribute to the hypertensive blood pressure levels in acromegalic patients. However, the multifocal expression of growth hormone has also a

number of other effects resulting in progressive glomerulosclerosis after induction of mesangial cell growth. (30)

In addition to hypertension, other risk factors of cardiovascular disease such as atherosclerosis have been investigated: Lipoproteins are the macromolecular transporters of non-polar lipids. The major high density lipoprotein (HDL) associated apolipoprotein is *apolipoprotein AI*. Plasma HDL concentrations as well as apoAI levels have been shown to be inversely correlated to the development of premature coronary heart disease. As its major apolipoprotein constituent, apoAI plays a central role in HDL assembly. The human apoAI gene was transferred into the atherosclerosis susceptible inbred mouse strain C57BL76. This transgene lead to a 2-fold increase in apoAI and HDL. Similarly, apolipoprotein E was expressed in transgenic mic (32). Although these mice were fed a high fat diet, they were markedly protected from atherosclerotic plaques. In other experiments, high levels of the *low density lipoprotein (LDL) receptor* was expressed using a receptor cDNA under the control of a metallothionein promoter. These mice cleared LDL from blood 8-times faster than normally. The transfer of LDL receptors to patients with known genetic LDL receptor defects as in familial hypercholesterolemia may be an approach to replace defective receptor function (33).

Transgenic animals in pulmonary disease

Most animal models which are used to mimic human disease are based on lesions which are applied to the adult animal and then the course of disease or the effect of treatment is analysed over time. Whereas animal models applicable to chronic and degenerative disease processes is less frequent developed. New animal models for chronic human disease may be generated by transgenic animals either by overexpression and excess function of a particular gene or by disruption of a functional gene. The success of these approaches depends on the extent to which a singular gene is indeed responsible for a disease. The investigation of *cystic fibrosis* the most common autosomal inherited disease, is a paradigm for these problems. The defective gene in cystic fibrosis patients has been identified as *Cfr*, which encodes an ion channel at the cell membrane. By homologous recombination, several groups succeeded to

disrupt the *Cfr* gene (34-36). All animal lines developed symptoms of cystic fibrosis. Although all these experiments created null mutations, the time course and the pattern of tissue involvement differed between the lines. These null mutation mice may be used for further studies on the *Cfr* gene, by introducing mutations of the *Cfr* gene via the transgenic approach. Comparison of the different clinically important mutations compromising the *Cfr* gene may clarify the tissue specific pattern of tissue involvement. Beyond that, these mice are excellent models to develop gene therapeutic strategies. Somatic transgenesis by liposomal transfection led to expression of a functional *Cfr* gene in the lung of these transgenic mice with the disrupted ion channel. (7). Gene therapeutical trials are now underway in humans with cystic fibrosis to apply the an intact *Cfr* gene supplementing the defective non-functional *Cfr* gene.

Transgenic animals in neurodegenerative disorders

As for chronic lung disease, transgenic methodology is being used to generate animal models which represent important aspects of human neurodegenerative disorders. The focus of interest has been *Alzheimer's disease*, where beta-amyloid, derived from the amyloid precursor protein, is chronically deposited in senile plaques and along vessel walls. In some forms of familial Alzheimer's disease, mutations of the amyloid precursor protein have been identified to be responsible for the development of the disease. Expression of the amyloid precursor protein in transgenic mice has not led to a distinctive Alzheimer's disease phenotype in the brain of these animals, however, a carboxyterminal fragment of the amyloid protein precursor causes neurodegeneration *in vivo* (37). Whether this closely correlates to Alzheimer's disease in humans is not clear and further studies aimed at overexpression of other components of the senile plaques such as the microtubule-associated protein *tau* are under way to generate an animal model for the human disease.

Transgenic animals in oncology or immunology

In these fields, most of the research employing transgenic technology is focused on fundamental studies rather than clinical disease. Several studies are being performed to

clarify the regulation of oncogenes. In particular, how they are activated and by which pathways these oncogenes exert their carcinogenic potential. Transgenic animals have been used as specific research tools to study the different steps in tumor development leading to a better understanding of the tumor-host interaction, tumor growth and angiogenesis and metastatic seeding. The analysis of these processes may lead to new therapeutic strategies, which could interrupt tumor development at the different stages. Similarly, the understanding of the immune system, the cytokine network and the host versus graft reaction in transplantation have been enhanced by transgenic approaches (38). The interaction of the cytokine network with antibody formation and B cell activity has been studied by "knock out" experiments. Gene targeting was used to disrupt genes involved in B and T cell differentiation (38,39). The role of cytokines and of differentiation factors in autoimmune and inflammatory disease has been investigated in several transgenic models. Overexpression of *human tumour necrosis factor alpha* in transgenic mice led to the development of chronic arthritis and systemic tumor necrosis factor-mediated disease (40). As a sign of chronic arthritis, accumulation of polymorphonuclear cells, synovial thickening and finally synovial hyperplasia and pannus, eroding the cartilage was observed. Similarly "knock out" of *transforming factor β* led to chronic and diffuse inflammation (41). How these experiments truly represent human syndromes of chronic inflammation remains to be elucidated, but they allow the investigation of the regulation of inflammatory processes.

Concluding remarks

Transgenic methodology has so rapidly developed that inclusion of all relevant data and publications is beyond the scope of this manuscript. However, it has been shown that transgenic methodology has spread into every field of biomedical research. Transgenic animals are being used to create new models for human disease, i) by generation of animal models which resemble the human condition as close as possible and ii) by in-detail analysis of the pathogenesis of human diseases under *in vivo* conditions. The further refinement of this technology from simple injection of foreign DNA into an oocyte to expression of large chromosomal regions and to the technique of embryonic stem cell manipulation underscores the fact that this technology is still in development.

Transgenic methodology, however, can not be considered in isolation: It is rather part of a major advancement gene technology has provided, extending into the fields of cell biology and molecular genetics. For examples, novel strategies in molecular genetics allow to identify the causative genes for disease and pathophysiological processes. In return, these genes can then be tested in transgenic animals on the functional and cellular level. In the future, these technologies can act in concert to develop new therapeutic strategies for human diseases and are therefore of interest for clinical applications. The methodological expansion, however, requires collaborative efforts of scientists and transfer of know-how between specialised laboratories more than ever.

REFERENCES

1. Gordon, J.W. & Ruddle, F.H. Integration and stable germ line transmission of genes injected into the mouse pronucleus. *Science* 1981; 214: 1244-1246.
2. Hammer, R.E., Maika, S.D., Richardson, J.A., Tang, J. & Taurog, J.D. Spontaneous inflammatory disease in transgenic rats expressing HLA-B27 and human b2m: An animal model of HLA-B27-associated human disorders. *Cell* 1990; 63: 1099-1112.
3. Mullins, J.J., Peters, J. & Ganten, D. Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. *Nature* 1990; 344: 541-544.
4. Ganten, D., Wagner, J., Zeh, K., Bader, M., Michel, J.-B., Paul, M., Zimmermann, F., Ruf, P., Hilgenfeldt, U., Ganten, U., Kaling, M., Bachmann, S., Fukamizu, A., Mullins, J.J. & Murakami, K. Species specificity of renin kinetics in transgenic rats harboring the human renin and angiotensinogen genes. *Proc Natl Acad Sci USA* 1992; 89: 7806-7810.
5. Hochi, S.-I., Ninomiya, T., Waga-Homma, M., Sagara, J. & Yuki, A. Secretion of bovine α -lactalbumin into the milk of transgenic rats. *Mol Reprod Develop* 1992; 33: 160-164.
6. Hogan, B., Costantini, F., & Lacy, E. Manipulating the mouse embryo, Cold Spring Harbor, New York 11724: Cold Spring Harbor Laboratory, 1986.
7. Hyde, S.C., Gill, D.R., Higgings, C.F. & Trezise, A.E.O. Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy. *Nature* 1993; 362: 250-255.
8. Nabel, E.G., Plautz, G. & Nabel, G.J. Site-specific gene expression in vivo by direct gene transfer into the arterial wall. *Science* 1990; 249: 1285-1288.
9. Zhu, N., Liggitt, D., Liu, Y. & Debs, R. Systemic gene expression after intravenous DNA delivery into adult mice. *Science* 1993; 261: 209-211.
10. Mulligan, R.C. The basic science of gene therapy. *Science* 1993; 260: 926-932.

11. Capecchi, M.R. Altering the genome by homologous recombination. *Science* 1989; 244: 1288-1292.
12. Jaenisch, R., Jahner, D., Nobis, P., Simon, T., Lohler, J., Harbers, K. & Grotkopp, D. Chromosomal position and advance of retroviral genomes inserted into germ line of mice. *Cell* 1981; 24: 519.
13. Hasty, P., Ramirez-Solis, R., Krumlauf, R. & Bradley, A. Introduction of a subtle mutation into the Hox-2.6 locus in embryonic stem cells. *Nature* 1991; 350: 243-246.
14. Grosveld, F., Blom van Assendelft, G., Greaves, D. & Kollias, G. Position-independent, high-level expression of the human b-globin gene in transgenic mice. *Cell* 1987; 51: 975-986.
15. Greaves, D., Wilson, F., Lang, G. & Kioussis, D. Human CD23-flanking sequences confer high-level T cell specific, position-independent expression in transgenic mice. *Cell* 1989; 51: 979-986.
16. Izzauralde, E., Mirkovitch, J. & Laemmli, U.K. Interaction of DNA with nuclear scaffolds *in vitro*. *J.Mol.Biol.* 1988; 200: 111-125.
17. Stief, A., Winter, D.M., Stratling, W.H. & Sippel, A.E. A nuclear DNA attachment element mediates elevated and position-independent gene activity. *Nature* 1989; 341: 343-345.
18. Burke, D., Carle, G. & Olson, M. Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. *Science* 1987; 236: 808-811.
19. Schedle, A., Beerman, F., Thies, E., Montoliu, L., Kelsey, G. & Schutz, G. Transgenic mice generated by pronuclear injection of a yeast artificial chromosome. *Nucleic Acids Res.* 1992; 20: 3073-3077.
20. Habener, J.F., Cwikel, B.J., Hermann, H., Hammer, R.E., Palmiter, R.D. & Brinster, R.L. Metallothionein-vasopressin fusion gene expression in transgenic mice. *J Biol Chem* 1989; 264: 18844-18852.
21. Steinhelper, M.E., Cochrane, K.L. & Field, L.J. Hypotension in transgenic mice expressing atrial natriuretic factor fusion genes. *Hypertension* 1990; 16: 301-307.
22. Jeunemaitre, X., Soubrier, F., Kotelevtsev, Y.V., Lifton, R.P., Williams, C.S., Charu, A., Hunt, S.C., Hopkins, P.N., Williams, R.R., Lalouel, J. & Corvol, P. Molecular basis of human hypertension: role of angiotensinogen. *Cell* 1992; 71: 169-180.
23. Hilbert, P., Lindpaintner, K., Beckmann, J.S., Serikawa, T., Soubrier, F., Dubay, C., Cartwright, P., DeGouyon, B., Julier, C., Takahasi, S., Vincent, M., Ganten, D., Georges, M. & Lathrop, G.M. Chromosomal mapping of two genetic loci associated with blood-pressure regulation in hereditary hypertensive rats. *Nature* 1991; 353: 521-529.
24. Wagner, J., Zeh, K. & Paul, M. Transgenic rats in hypertension research. *J Hypertens* 1992; 10: 601-605.
25. Kimura, S., Mullins, J.J., Bunnemann, B., Metzger, R., Hilgenfeldt, U., Zimmermann, F., Jacob, H., Fuxe, K., Ganten, D. & Kaling, M. High blood pressure in transgenic mice carrying the rat angiotensinogen gene. *EMBO J* 1992; 11: 821-827.

26. Takahashi, S., Fukamizu, A., Hasegawa, T., Yokoyama, M., Nomura, T., Katsuki, M. & Murakami, K. Expression of the human angiotensinogen gene in transgenic mice and transfected cells. *Biochem Biophys Res Commun* 1991; 180: 1103-1109.
27. Ohkubo, H., Kawakami, H., Kakehi, Y., Takumi, T., Arai, H., Yokota, Y., Iwai, M., Tanabe, Y., Masu, M., Hata, J., Iwao, H., Okamoto, H., Yokoyama, M., Nomura, T., Katsuki, M. & Nakanishi, S. Generation of transgenic mice with elevated blood pressure by introduction of the rat renin and angiotensinogen genes. *Proc Natl Acad Sci USA* 1990; 87: 5153-5157.
28. Fukamizu, A., Sugimura, K., Takimoto, E., Sugiyama, F., Seo, M.S., Takahashi, S., Hatae, T., Kajiwar, N., Yagami, K. & Murakami, K. Chimeric renin angiotensin system demonstrates sustained increase in blood pressure of transgenic mice carrying both human renin and human angiotensinogen genes. *J Biol Chem* 1993; 268: 11617-11621.
29. Dilley, R.J. & Schwartz, S.M. Vascular remodeling in the growth hormone transgenic mouse. *Circ Res* 1989; 65: 1233-1240.
30. Doi, T., Striker, L.J., Gibson, C.C., Agodoa, L.Y., Brinster, R.L. & Striker, G.E. Glomerular lesion in mice transgenic for growth hormone and insulin-like growth factor-I: relationship between increased glomerular size and mesangial sclerosis. *Am J Pathol* 1990; 137: 541-552.
31. Swanson, M.E., Hughes, T.E., St.Denny, I., France, D.S., Paterniti, J.R., Tapparelli, C., Geller, P. & Burki, K. High level expression of human apolipoprotein A-I in transgenic rat raises total serum high density lipoprotein cholesterol and lowers rat apolipoprotein A-I. *Transgenic Research* 1992; 1: 142-147.
32. Shimano, H., Yamada, N., Katsuki, M., Shimada, M., Gotoda, T., Harada, K., Murase, T., Fukazawa, C., Takaku, F. & Yazaki, Y. Overexpression of apolipoprotein E in transgenic mice: marked reduction in plasma lipoproteins except high density lipoprotein and resistance against diet-induced hypercholesterolemia. *Proc Natl Acad Sci USA* 1992; 89: 1750-1754.
33. Hofmann, S.L., Russell, D.W., Brown, M.S., Goldstein, J.L. & Hammer, R.E. Overexpression of low density lipoprotein (LDL) receptor eliminates LDL from plasma in transgenic mice. *Science* 1988; 239: 1277-1281.
34. Snouwaert, J.M., Brigman, K.K., Latour, A.M., Malouf, N.N., Boucher, R.C., Smithies, O. & Koller, B.H. An animal model for cystic fibrosis made by gene targeting. *Science* 1992; 257: 1083-1088.
35. Dorin, J.R., Dickinson, P., Alton, E.W.F.W., Smlth, S.N., Geddes, D.M., Stevenson, B.J., Kimber, W.L., Fleming, S., Clarke, A.R., Hooper, M.L., Anderson, L., Beddington, R.S. & Proteous, D.J. Cystic fibrosis in the mouse by targeted insertional mutagenesis. *Nature* 1992; 359: 211-215.
36. College, W.H., Ratcliff, R., Foster, D., Williamson, R. & Evans, M.J. Cystic fibrosis mouse with intestinal obstruction. *Lancet* 1992; 340: 680
37. Neve, R.L., Kozlowski, M.R., Kammerscheidt, A., & Hohmann, C. The carboxyterminal fragment of the Alzheimer amyloid protein precursor causes neurodegeneration *in vivo*. In: *Transgenic animals as model system for human diseases*. eds. E.F. Wagner & F. Theuring, Berlin: Springer-Verlag, 1993; 57-72.

38. Parham, P. Some savage cuts in defence. *Nature* 1990; 344: 709-711.
39. Rajewsky, K. A phenotype or not: targeting genes in the immune system. *Science* 1992; 256: 483
40. Keffer, J., Probert, L., Cazlaris, H., Georgopoulos, S., Kaslari, E., Kioussis, D. & Kollias, G. Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. *EMBO J* 1991; 10: 4025-4031.
41. Shull, M.M., Ormsby, I., Kier, A.B., Pawlowski, S., Diebold, R.J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., Annunziata, N. & Doetschman, T. Targeted disruption of the mouse transforming growth factor- β 1 gene results in multifocal inflammatory disease. *Nature* 1992; 359: 693-699.

Submitted: 11/1/94

Accepted: 12/9/94



The Royal Society

Proceedings: Biological Sciences

Volume 251 Pages 165–267 Number 1332 22 March 1993

Proceedings of the Royal Society of London SERIES B

Transgenic birds from transformed primordial germ cells

LORRAINE VICK, YING LI AND K. SIMKISS†

Department of Pure and Applied Zoology, School of Animal and Microbial Sciences, University of Reading, Reading RG6 2AJ, U.K.

[Plate 1]

SUMMARY

Primordial germ cells (PGCs) are the progenitor cells for the gametes. They can be obtained from avian embryos by dissociating the germinal crescent region, where they accumulate, or by sampling the blood at the time of their migration to the gonad. PGCs were obtained from these sources and transfected with defective retroviruses. These manipulated cells were injected into recipient embryos to form chimaeras which grew to sexual maturity and produced offspring, some of which contained the foreign DNA. This is the first example of the direct use of PGCs to produce transgenic offspring.

1. INTRODUCTION

Several attempts have been made to introduce foreign DNA into the avian genome to form a transgenic bird. These have included coating the sperm with DNA before fertilization (Gavora *et al.* 1991), attempting microinjection into the male pronucleus of the fertilized egg (Perry *et al.* 1991), and injecting replication-competent (Salter *et al.* 1986) or replication-defective (Bosselman *et al.* 1990) retroviruses into the blastoderm. At the present time, only the infection of the blastoderm by retroviruses has been successful in introducing exogenous genes into the chicken germ line. There are several reasons for these difficulties but the most obvious relate to the avian system of reproduction in which the presence of a large yolky egg with many supernumerary pronuclei (Eyal-Giladi & Kochav 1976) and the fact that the embryo develops to a stage of about 50 000 cells before it is laid (Spratt & Haas 1960) make the standard techniques difficult to apply. To overcome these constraints attention has shifted towards the possibility of producing chimaeric animals as vehicles for gaining access to the avian genome. The most obvious way of doing this is to use primordial germ cells (PGCs) because these are the progenitors of the gametes. Thus, if these cells could be isolated, transformed with foreign DNA and introduced into recipient embryos, some of them should populate the gonads to produce a chimaeric organ. Gametes derived from such cells would produce transgenic animals in the subsequent generation.

In previous work we have shown that it is possible to transfer PGCs from one chick embryo to another to form animals with such chimaeric gonads (Simkiss *et al.* 1989). Subsequently we demonstrated that it was

possible to transfect these donor PGCs with a defective retrovirus so that the foreign DNA was incorporated into the gametes (Simkiss *et al.* 1990). In both these experiments we took advantage of two features of avian development that are unusual among vertebrates. In birds, and a few reptiles, the primordial germ cells accumulate very early in development at an extra-embryonic site in front of the head at the junction of the area pellucida and the area opaca. In the domestic fowl this so-called germinal crescent forms after 2 d of incubation. After about 2½ d of incubation these cells show a second unusual feature in that they migrate via the developing blood system, and are transported to the germinal ridge where they settle in what is to become the definitive gonad (Nieuwkoop & Satauraya 1979). Primordial germ cells can, therefore, be obtained either directly from the germinal crescent region or indirectly by taking carefully timed blood samples from the stage 16 embryo (Al Thani & Simkiss 1991). These cells can then be introduced into recipient embryos where they contribute to the formation of a chimaeric gonad.

The present work was undertaken to quantify some of these processes and to establish that it is possible to use PGCs to produce transgenic birds.

2. MATERIALS AND METHODS

A retrovirus-free strain (line zero) of White Leghorn fowl (Astrin *et al.* 1979) was used in most of these experiments. When other birds were required an in-bred line of Rhode Island Reds was used.

Fertile eggs were incubated at 37.5 °C and 70% relative humidity in a forced air incubator (Brinsea Ltd). Embryos were exposed by removing a small piece of shell, and their stage of development was determined from the morphological criteria of Hamburger & Hamilton (1951). Germinal cres-

† To whom correspondence should be addressed.

cents were obtained from embryos at stage 11 (roughly 40 h incubation) and blood samples were taken from embryos at stage 15 (roughly 55 h incubation). Germinal crescents were dissociated in No-zyme (JRH Biosciences, Sera-Lab Ltd) for 15 min, washed in Hanks balanced salt solution and dispersed in a syringe. The cell suspension was centrifuged at 1000 r.p.m. (Eppendorf 5402) for 30 s and any yolk cells and debris were removed. Primordial germ cells were resuspended in 200 µl Hanks saline, and a 5 µl sample was spun onto a slide (Cytospin 3 Shandon Scientific Ltd). Blood samples were taken from the dorsal aorta of suitable embryos by using a fine needle and syringe. A 5 µl sample was smeared onto a slide for cell counting. The viability of cell preparations was determined using erythrosin B (5 g l^{-1}) for 2 min as a stain exclusion test.

Two defective retroviruses were used to transform pGCs. The construction of a defective spleen necrosis virus (SNO21) has been described by Meyers *et al.* (1991). Some clones of this retrovirus showed extensive reorganization in the helper cell line, and a second vector based on a defective avian leukosis virus (NLB) was therefore used in later work. The construction of this retrovirus is given in detail by Cosset *et al.* (1991). It consists of the long terminal repeat (LTR) sequences of the Rous associated virus (RAV-2) with *neo* & *lacZ* genes inserted between the *gag* initiator codon and the 3' end of the *env* gene (figure 2). The defective retrovirus was screened periodically to ensure that no replication-competent virions were produced. Transfection of cells was done using equal volumes of cell suspension and replication defective retrovirus, containing roughly 10^6 virions cm^{-3} which were mixed on a rotating platform for 20 min at 37 °C. Transfected cells were washed and 5 µl samples injected into the vasculature of stage 15 embryos by using glass micropipettes produced with a Narishige micropipette puller and microforge. The window in the shell was covered with Micropore surgical tape (3M Health-care) and the eggs were subsequently incubated normally. Viability was checked by routine candling and selected hatchlings were raised to sexual maturity.

In the NLB experiments, line zero (White Leghorn) pGCs were transfected with the defective retrovirus, and these cells were then introduced into Rhode Island Red recipient embryos. Hatchlings were raised to sexual maturity and crossed with White Leghorns.

DNA was prepared from tissue samples by homogenizing and digesting them with proteinase K and RNase before phenol-chloroform extraction and ethanol precipitation. Samples of DNA (0.1–0.5 µg) were digested with restriction endonucleases at 37 °C for 6–8 h and run on electrophoresis gels with *Hind* III digested bacteriophage λ as molecular mass markers. Southern blots were prepared and DNA identified by using [^{32}P]-labelled probes produced by nick translation of the original plasmids (Maniatis *et al.* 1982). In the case of SNO21 this screening was based upon detecting a 6.7 kilobase fragment of DNA that hybridized to the [^{32}P] probe. This fragment was predicted from the original map of the vector (Meyers *et al.* 1991) which contained two *Sma* I cutting sites at this position, and this was confirmed by transfecting quail fibroblasts (QT 35 line) with vSNO21 and probing the DNA. The NLB vector contains two unique *Bam* HI cutting sites 4.5 kilobase pairs apart and this fragment was identified by hybridizing to the equivalent [^{32}P]-labelled plasmid.

3. RESULTS

The primordial germ cells of the fowl embryo are typically 15–20 µm in diameter with a large eccentric

nucleus (8 µm diameter) and numerous yolk and glycogen granules in the cytoplasm (Simkiss 1991). The mean number of pGCs per germinal crescent of a stage 11 line 0 White Leghorn embryo was 95 ± 8 , and the viability of the pGCs decreased to roughly 68% during their isolation from the germinal crescent. This was attributed to damage during their extraction (table 1). pGCs from the blood averaged 11 ± 3 per 10 µl sample.

Opening eggs incubated for 2 d resulted in roughly 20% mortality of embryos and intravascular injection caused a further 40% mortality (figure 1). To test whether donor pGCs had been incorporated into their gonads, embryos were sampled at 18 d, as this corresponds with the maximum number of gonocytes.

DNA derived from the vSNO21-transfected cells was

Table 1. *Viability of pGCs derived from germinal crescents*

stage of procedure	viability (%)
treatment with No-zyme	93
tissue dissociation in syringe	84
centrifugation and resuspension	68
transfection with vector	54

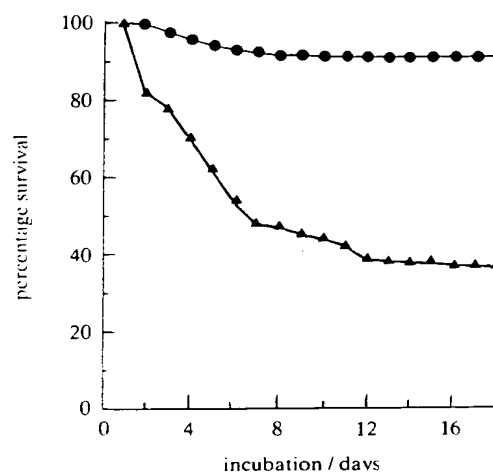


Figure 1. Percentage survival of control (circles) and recipient (triangles) embryos after being injected with transformed pGCs ($n = 200$ for each data set).

Table 2. *Effect of source of pGCs on chimera production in 18 d embryos by using SNO21*

source	number of embryos	number with positive DNA
blood	69	2 (3%)
germinal crescent	22	5 (23%)

Table 3. *Number of offspring analysed before a transgenic chick was obtained*

bird	retrovirus	number of embryos	number transgenic
cock 1	SNO21	56	1 (2%)
cock 2	NLB	24	1 (4%)



Figure 3. The chimaeric cock 2 (centre) which contained some White Leghorn PGCs transfected with the NLB defective retrovirus. Most of this bird's offspring appeared like normal Rhode Island Red birds (left) but he also produced some offspring (right) with a White Leghorn phenotype (F_1 bird 24) that were transgenic. The Southern blots in figure 2 are from these birds.

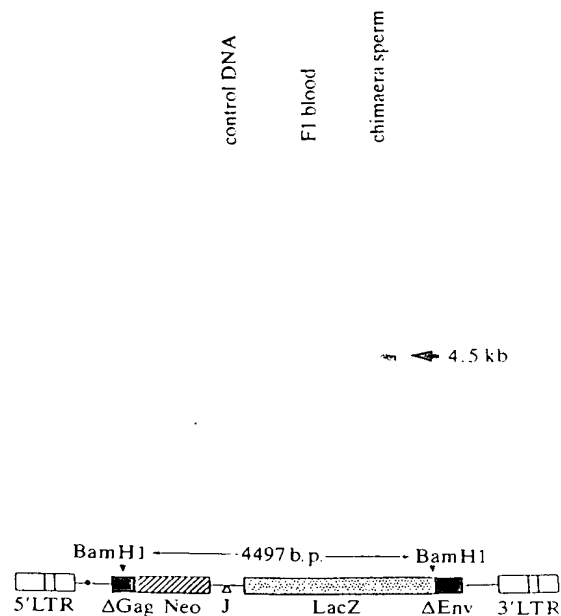


Figure 2. Southern plots of the DNA from the sperm of the chimaeric bird (right, cock 2) and the blood of one of his offspring (centre, F1 bird 24) which is transgenic. The band at 4.5 kb corresponds with the *Bam* H1 fragment of the NLB vector shown in map form at the bottom. Both the sperm of the chimaera and the transgenic offspring contain this foreign DNA which is absent from the DNA of control birds (left).

found in roughly 3% of the embryos injected with pGCs-containing blood samples and in 23% of the embryos injected with germinal crescent samples (table 2). Hatchlings were produced from similarly manipulated embryos and five cockerels were raised to sexual maturity. Not all of these produced good samples of sperm for artificial insemination but two cockerels produced $2-3 \times 10^6$ sperm cm^{-3} , and the sperm contained vector DNA. These were therefore selected for breeding experiments. In the case of cock 1, 56 hatchlings were produced and their blood screened for vector DNA before a positive specimen was obtained. With cock 2, which contained NLB-treated pGCs, the 24th chick to be produced was transgenic (table 3). In this latter case the Rhode Island Red (RIR) chimaeric cock was mated with a White Leghorn (WL) hen. This produced two types of offspring. The most common were initially white with black spots; these chicks turned brown in about 3 months. These are the characteristic offspring of a RIR \times WL cross in our flock. Occasionally, however, a pure White Leghorn was produced (figure 3) which was also shown to be a transgenic bird (figure 2).

4. DISCUSSION

The aim of this work was to demonstrate the feasibility of using primordial germ cells as a means of gaining access into the avian genome. Foreign DNA was introduced into these cells by using two defective retroviruses as these systems have a high efficiency for

infecting cells with single copies of the foreign DNA (Varmus 1988). Two sources of pGCs were used representing cells at different stages of their normal migratory activity.

Primordial germ cells that are obtained from embryonic blood are already in transit to the germinal ridge but they produced chimaeric embryos in only 3% of these experiments. This is probably because the migration of these cells occurs as a short temporal pulse (Al-Thani & Simkiss 1991) that may be missed in individual donor embryos. A much higher rate of chimaera production (23%) was obtained by extracting the primordial germ cells from the germinal crescent before their vascular migration (table 2). Clearly it is the number of primordial germ cells that can be obtained rather than their 'cellular maturity' that determines the success rate in forming these embryos. Chimaeric hatchlings were produced, however, from both blood-derived and germinal crescent-derived pGCs.

The initial experiments used blood-derived pGCs transfected with vSNO21 and injected into line zero embryos. Four cockerels were hatched and raised to sexual maturity from these experiments, and one of them (cock 1) was subsequently used for breeding experiments. Because this cock was a chimaera, those offspring that were derived from its transfected cells should be transgenics, whereas those derived from its own cells would be normal. The production of one transgenic in 56 hatchlings is roughly in keeping with these expectations. The blood source that was used to form this chimaera contained roughly 1-2 pGCs per microlitre before dilution with the vector; the injection volume was roughly 5 μl . Thus 3-5 pGCs would have been injected into a recipient embryo with 200-300 endogenous pGCs. Assuming a high rate of transfection and that the chances of colonizing the germinal ridge are equal for injected and endogenous pGCs, this would produce transgenics in approximately 1-4% of cases, i.e. in the range found. Despite this it was felt that the instability of the defective retrovirus vSNO21 and the absence of an accurate map for this construct made these results inconclusive.

A second series of experiments was therefore done using pGCs from the germinal crescent of a line zero White Leghorn embryo which were transfected with the vector NLB and then injected into a Rhode Island Red recipient. A cockerel produced from this experiment was raised to maturity. Semen from this animal (cock 2), gave a 4.5 kilobase (kb) fragment on Southern blots of *Bam* H1 cut DNA, showing that it contained transfected sperm (figure 2). Crossing this bird with a White Leghorn hen produced two types of offspring. The most common were the characteristic offspring of a cross between a White Leghorn and a Rhode Island Red, but occasionally a pure White Leghorn phenotype was obtained. These were clearly the offspring of sperm derived from the White Leghorn pGCs that had been injected into the Rhode Island Red embryo. The DNA from a blood sample from this white F1 cockerel was analysed and shown to contain the 4.5 kb fragment of vector DNA so that both traditional genetics and DNA screening identified this

bird as being a transgenic derived from the transfected donor PGCs that were used to form the chimaeric cockerel.

These experiments demonstrate, therefore, that it is possible to obtain transfected PGCs that can be used to produce chimaeric birds. The foreign DNA will only be present in some of the germ cells from these birds, but by using PGCs from different strains it is possible to identify such transgenic birds easily. Clearly, the next stage in the improvement of this approach to producing transgenic birds is to increase the efficiency of the procedure, either by sterilizing the recipient embryos (Aige-Gil & Simkiss 1991) or by increasing the input of transformed PGCs. It will be apparent, however, that studying the chimaeric birds that are used in this approach will also provide additional information on their reproductive biology. By measuring the number of copies of vector DNA in the sperm of chimaeric birds it will be possible to quantify the ratio of transfected to normal sperm and thus the probability of producing transgenic offspring. Furthermore, because retroviruses insert into their host DNA in a largely random fashion, it is also possible to cut the vector DNA so as to release a variety of junction fragments that identify each of the individual PGCs that have settled in the gonad. Thus information on both the number of PGCs and their relative fecundity can be obtained from these experiments. At the present time it appears that the number of sperm carrying foreign DNA varies between ejaculates, suggesting that particular regions of the testis may be intermittently involved: this is currently being investigated.

The technique of using PGCs to produce gonadal chimaeras and transgenic offspring may also have other implications. There have recently been a number of suggestions that PGCs may be capable of producing embryonic stem (ES) cells in culture (Resnick *et al.* 1992; Matsui *et al.* 1992). If these results can be extended to *in vivo* studies they may provide important techniques for studying the whole process of embryonic differentiation.

We thank Dr N. Bumstead of AFRC Institute of Animal Health and Dr G. Verdier, Dr V.-G. Nigon, Dr Y. Chebloune and Dr C. Legras of Laboratoire de Biologie Cellulaire, Université Claude Bernard for providing us with defective retroviruses. This work was supported by AFRC grants (AT45/521, AT45/600), by Merck Research Laboratories and by an Anglo-French Alliance award (PN92.214).

REFERENCES

- Aige-Gil, V. & Simkiss, K. 1991 Sterilization of avian embryos with busulphan. *Res. Vet. Sci.* **50**, 139-144.
- Al-Thani, R. & Simkiss, K. 1991 Effects of an acute *in vivo* application of concanavalin A on the migration of avian primordial germ cells. *Protoplasma* **161**, 52-57.
- Astrin, S. M., Buss, E. G. & Hayward, W. S. 1979 Endogenous viral genes are non-essential in the chicken. *Nature, Lond.* **282**, 339-341.
- Bosselman, R. A., Hsu, R.-Y., Briskin, M. J., Boggs, T., Hu, S., Nicolson, M., Souza, L. M., Schultz, J. A., Rishell, W. & Stewart, R. G. 1990 Transmission of exogenous genes into the chicken. *J. Reprod. Fert. (Suppl.)* **41**, 183-195.
- Cosset, F.-L., Legras, C., Thomas, J.-L., Molina, R.-M., Chebloune, Y., Faure, C., Nigon, V.-M. & Verdier, G. 1991 Improvement of avian leukosis virus (ALV)-based retrovirus vectors by using different cis-acting sequences from ALVs. *J. Virol.* **65**, 3388-3394.
- Eyal-Giladi, H. & Kochav, S. 1976 From cleavage to primitive streak formation. A complimentary normal table and a new look at the first stages of the development of the chick. 1. General morphology. *Dev. Biol.* **49**, 321-337.
- Gavora, J. S., Benkal, B., Sasada, H., Cantwell, W. J., Fiser, P., Teather, R. M., Nagai, J. & Sabour, M. P. 1991 An attempt at sperm-mediated gene transfer in mice and chickens. *Can. J. Anim. Sci.* **71**, 287-291.
- Hamburger, V. & Hamilton, H. 1951 A series of stages in the development of the chick embryo. *J. Morph.* **88**, 49-92.
- Hughes, G. C. 1963 The population of germ cells in the developing female chick. *J. Embryol. exp. Morph.* **11**, 513-536.
- Maniatis, T., Fritsch, E. F. & Sambrook, J. 1982 *Molecular cloning: a laboratory manual*. New York: Cold Spring Harbor Laboratory Press.
- Matsui, Y., Zsebo, K. & Hogan, B. L. M. 1992 Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell*, **70**, 841-847.
- Meyers, N. L., Booth, S. C., Bumstead, N., Vick, L. & Simkiss, K. 1991 Development and testing of a packaging cell line for avian retroviral vectors. *Archs Virol.* **119**, 257-264.
- Nieuwkoop, P. D. & Satawrya, L. A. 1979 *Primordial germ cells in the chordates*. Cambridge University Press.
- Perry, M. M., Morrice, D., Hettle, S. & Sang, H. 1991 Expression of exogenous DNA during the early development of the chick embryo. *Wilhelm Roux Arch. dev. Biol.* **200**, 312-319.
- Resnick, J. L., Bixler, L. S., Cheng, L. & Donovan, P. J. 1992 Long term proliferation of mouse primordial germ cells in culture. *Nature, Lond.* **259**, 550-551.
- Salter, D. W., Smith, E. J., Hughes, S. H., Wright, S. E., Fadly, A. M., Witter, R. L. & Crittenden, L. B. 1986 Gene insertion into the chicken germ line by retroviruses. *Poultry Sci.* **65**, 1445-1458.
- Simkiss, K. 1991 Primordial germ cells and the scope for genetic manipulation using embryos. In *Avian incubation* (ed. S. Tullett), pp. 125-136. London: Butterworth-Heinemann.
- Simkiss, K., Rowlett, K., Bumstead, N. & Freeman, B. M. 1989 Transfer of primordial germ cell DNA between embryos. *Protoplasma* **151**, 164-166.
- Simkiss, K., Vick, L., Luke, G., Page, N. & Savva, D. 1990 Infection of primordial germ cells with defective retroviruses and their transfer to the developing embryo. *4th World Congr. Gen. Appl. Livestock Prod.* **116**, 111-114.
- Spratt, N. T. & Haas, H. 1960 Morphogenetic movements in the lower surface of the incubated and early chick blastoderm. *J. exp. Zool.* **144**, 139-157.
- Varmus, H. 1988 Retroviruses. *Science, Wash.* **240**, 1427-1435.

Received 26 November 1992; accepted 14 December 1992

Plate 1 was printed by George Over Ltd., Rugby and London, U.K.